

# **Effects of long-term hypothyroidism in the morphology and synaptic organization of cerebellar ectopic granule cells**

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**Summary.** Abundant ectopic granule cells scattered in the cerebellar molecular layer have been observed in 30-day-old hypothyroid rats. Their morphological features indicate that they must be regarded as mature heterotopic cells arrested during their migration towards the granular layer. As their impoverished dendritic trees are identical to those seen in controls, it is unlikely that the lack of thyroid hormones played a major role in the deficient dendritic outgrowth. The study of 180-day-old hypothyroid rats revealed that although ectopic granule cells remained quite numerous, their number per unit surface was lesser than in the 30-day-old hypothyroid group. This finding may be related to the capacity displayed by heterotopic neurons to establish synaptic contacts with the components of the molecular layer. This was inferred by the presence of a peculiar synaptic cell investment formed by axosomatic and somatodendritic contacts in 180-day-old hypothyroid rats which shows that the surviving ectopic granule cells manage to adapt to an adverse milieu.

**Key words:** Hypothyroidism Ectopic granule cells, Cerebellum, Rat

## **Introduction**

Isolated ectopic granule cells (EGCs) are a common finding in the cerebellar molecular layer of rodents (Chan-Palay, 1972; Spacek et al., 1973; Lafarga and Berciano, 1985). Their number increases dramatically after experimental manipulations causing incomplete damage with subsequent recovery of the external granular layer (EGL) (Ebels, 1972), as is the case of x-irradiation (Altman, 1973; Ebels and Peters, 1974), viral infections (Monjan et al., 1971) or following the

administration of cytostatic drugs (Nathanson et al., 1969; Shimada and Langman, 1970a, b). In addition, changes in the process of granule cell migration towards the internal granular layer were also implicated in the genesis of EGCs, as described in some genetic mutants (Landis, 1973; Mariani et al., 1977) and after chemically-induced degeneration of Bergman glia (Sotelo and Rio, 1980).

A retarded and prolonged differentiation of cells in the EGL (Balazs et al., 1971; Nicholson and Altman, 1972a; Legrand, 1982), together with a reduction in the rate of granule cell migration (Lauder, 1979), has been detected in hypothyroid rats. It has been postulated that these changes could underlie the marked transient depression observed during the process of granular layer cell acquisition and that this could be compensated by the elongation of the neurogenic period (Nicholson and Altman, 1972a; Lewis et al., 1976; Legrand, 1982).

Using stereological methods, we recently evaluated the effects of hypothyroidism on the number of cerebellar granule cells in 30-day-old rats (Madeira et al., 1986, 1988) and found that, although their numbers per unit surface and per unit volume of the granular layer were identical in controls and hypothyroids, as previously pointed out (Nicholson and Altman, 1972a; Clos and Legrand, 1973), their total number was reduced. Furthermore, during these studies, we noted a remarkable increase in the cellularity of the molecular layer of hypothyroid rats due to the presence of numerous mature EGCs.

We felt that it would be of interest to study the morphological features of this neuronal population located in an adverse milieu and whose development had occurred in the absence of thyroid hormones that actually deeply interfere with the outgrowth of neuronal processes (Ruiz-Marcos et al., 1979; Legrand, 1982). Besides, as EGCs show a great ability to adapt to different environments, as is inferred by the moulding of their dendritic trees and the formation of synapses (Sotelo and Rio, 1980; Lafarga and Berciano, 1985) even

in extraparenchymatous areas (Sievers et al., 1985), it was decided to extend this study to 180-day-old hypothyroid rats to evaluate whether these plastic potentialities remained present after longer periods of thyroid hormone deficiency.

## **Materials and methods**

### *Animals and treatment*

Sprague-Dawley male rats from the Colony of Gulbenkian Institute of Science (Oeiras) were used. The number of rats per litter ranged between 6 and 8. The 4 groups studied (30- and 180-day-old hypothyroid rats and respective age-matched controls) were formed from 6 different litters.

Thirty-day-old rats were rendered hypothyroid with a daily subcutaneous injection of propylthiouracil (PTU): 0.05 ml 0.2% PTU on days 0-10; 0.1 ml 0.2% PTU on days 11-20 and 0.1 ml 0.4% PTU on days 21-30 (Nicholson and Altman, 1972a). Animals were considered to be hypothyroids based on histological criteria.

Those rats allowed to survive until 180 days were treated as previously described until day 30. A thyroidectomy was then performed under nembutal anaesthesia. The completeness of the thyroidectomy was judged by the inspection and by the histological examination of serial sections of the trachea at necropsy (Magalhães and Magalhães, 1981).

Age-matched control animals were also obtained from different litters. Until 30 days of age they were daily injected subcutaneously with 0.1 ml of physiological saline. In the 180-day-old control rats the surgical steps accomplished for the thyroidectomy were carried out up to the exposure of the thyroid gland.

### *General Procedures*

The animals were anaesthetized with ether and transcardially perfused with a solution of 1% glutaraldehyde /1% paraformaldehyde in 0.12 M phosphate buffer at pH 7.2. The cerebella were removed and immersed for 2 hours in the perfusion solution.

Parasagittal sections of vermal lobules IV-VI (Larsell, 1952) were processed for electron microscopy. The cerebellar hemispheres were Golgi impregnated.

### *Electron Microscopy*

Sections were osmicated in a solution of 2% osmium tetroxide in 0.12 M phosphate buffer for 2 hours, block-stained with uranyl acetate, dehydrated in ethanols and epon embedded. Semithin sections (2 µm) were stained with toluidine blue and ultrathin sections double stained with uranyl acetate and lead citrate

### *Golgi impregnation*

The cerebellar hemispheres were post-fixed in the solution used for perfusion for a week, and Golgi

impregnated according to a method similar to that used by Stensaas (1967), with the modifications introduced by Eckenhoff and Rakic (1984). The hemispheres were immersed in 50 ml of a solution of glutaraldehyde (25%) 10 ml, formaldehyde (39%) 10 ml, potassium dichromate 5 g, dymethylsulfoxide 5-9 drops and distilled water to 100 ml for 72 h. The specimens were then transferred into 1.5% silver nitrate and stored in the dark for 3-5 days. The hemispheres were shelled in a paraffin block and sliced (100 µm thick) in parasagittal planes.

### *Quantitative and statistical analysis*

The number of EGCs per unit surface of molecular layer was calculated in the 30- and 180-day-old hypothyroid groups. From each animal five blocks were used. From each block 2 semithin sections were obtained and drawn at the camera lucida. EGCs were counted and the molecular layer area measured in a Mop-Videoplan.

In control groups EGCs were not quantified since too few cells were observed to allow a reliable study.

The data was analysed using the two-tailed nonparametric Mann-Whitney U-test. Differences were considered significant if  $p < 0.05$ .

## **Results**

### *Semithin section observations*

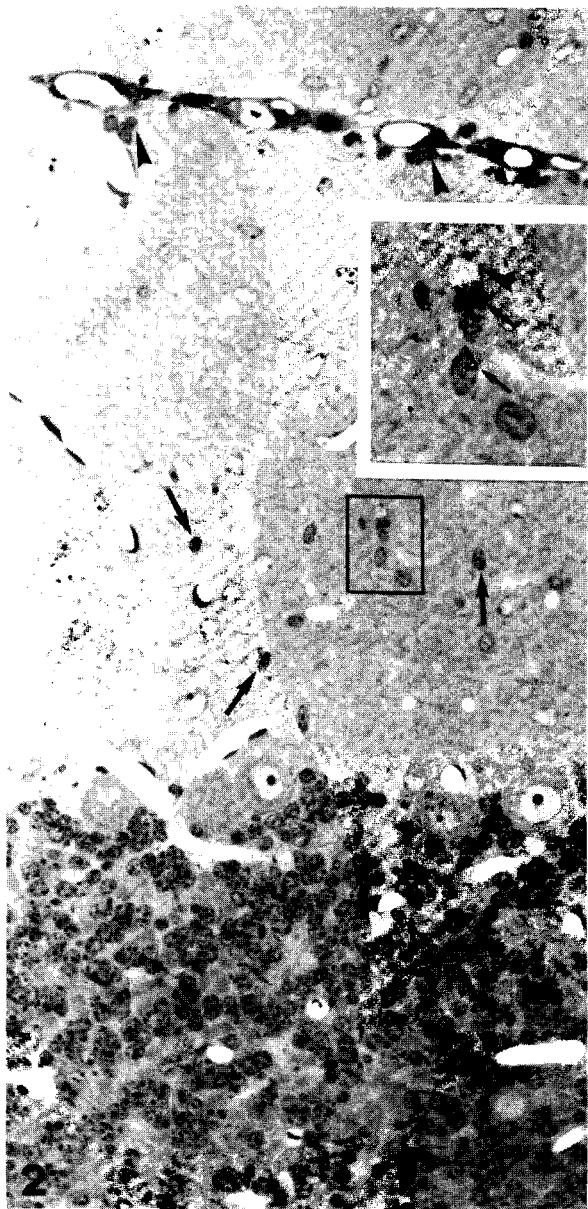
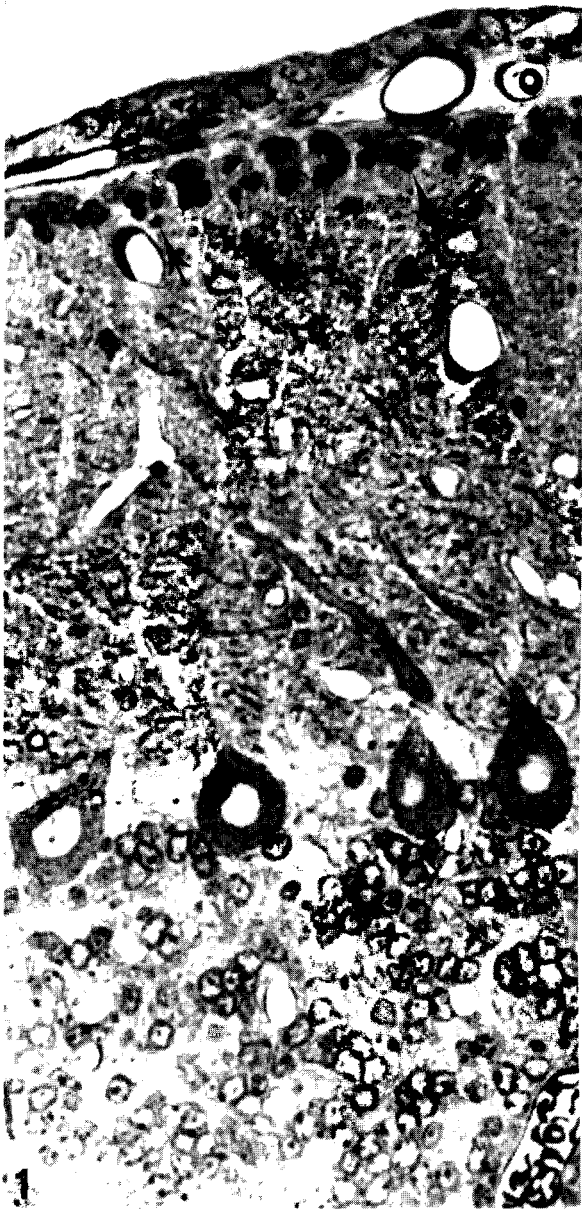
EGCs were discriminated from the other cells normally seen in the cerebellar molecular layer because their nuclei are characteristically smaller and electron denser, with the heterochromatin forming clumps close to the nuclear membrane (Figs. 1,2). No qualitative differences could be found between EGCs nuclei from hypothyroid and control rats.

In hypothyroid rats vestiges of the EGL (Figs. 1, 2) and small foci of arrested granule cells beneath the pial surface were a common finding, as opposed to controls where similar arrangements were never found. Radial glial fibres and bipolar granular profiles were rarely seen in control and hypothyroid rats. On the contrary, in the latter groups scattered EGCs were observed at all levels of the molecular layer, although most of them were situated in its inner third (Fig. 2).

A significant reduction ( $p < 0.05$ ) was found when the number per unit surface of EGCs from the 180-day-old hypothyroid group was compared with that of the 30-day-old hypothyroid group (Fig. 3).

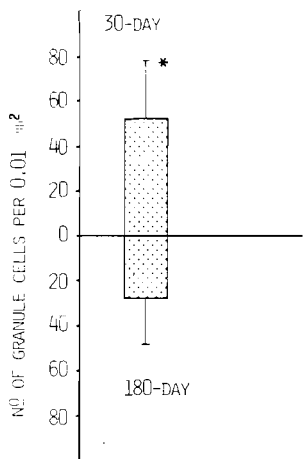
### *Golgi observations*

As opposed to what happened in the granular layer where granule cells are easily impregnated, with the Golgi method used the number of impregnated EGCs was scarce, specially in controls. Five impregnated cells were seen in control animals whereas 51 and 42 cells were seen in the 30- and 180-day-old hypothyroid rats respectively. Despite the reduced number of cells observed in controls no qualitative differences seem to

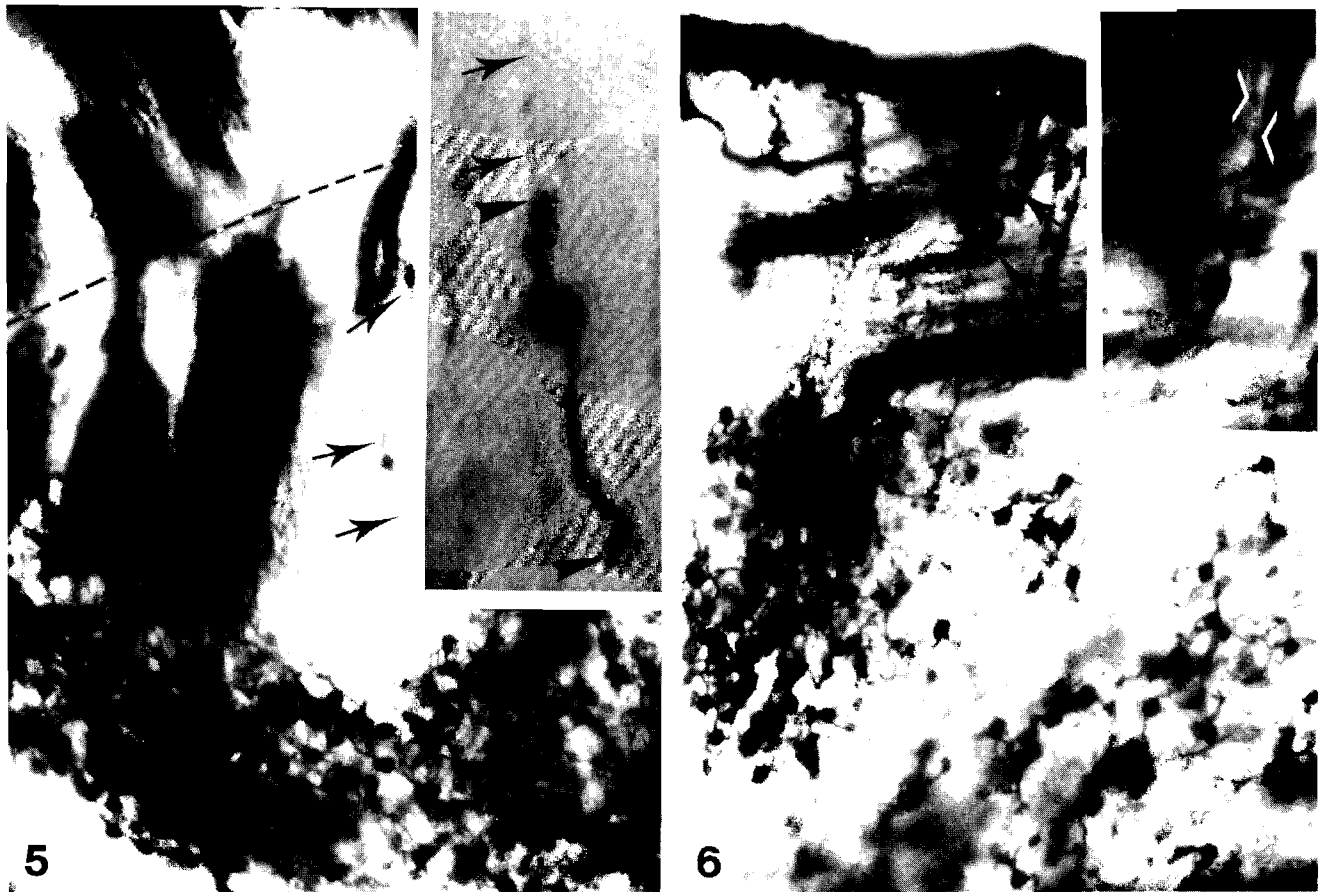


**Fig. 1.** External granular layer in a 30-day hypothyroid rat (arrows). Migrating granule cells are not seen in the molecular layer. Toluidine blue.  $\times 315$

**Fig. 2.** Ectopic granule cells (EGCs) in the molecular layer of a 180-day hypothyroid rat (arrows), more numerous in its inner third. Subpial EGCs are also recognized (arrow-heads).  $\times 195$ . Inset. Higher magnification of EGCs. Their round nuclei and peculiar chromatin disposition (arrows) allow an easy differentiation from the normal cellular components (arrow-heads) of the molecular layer. Toluidine blue.  $\times 315$



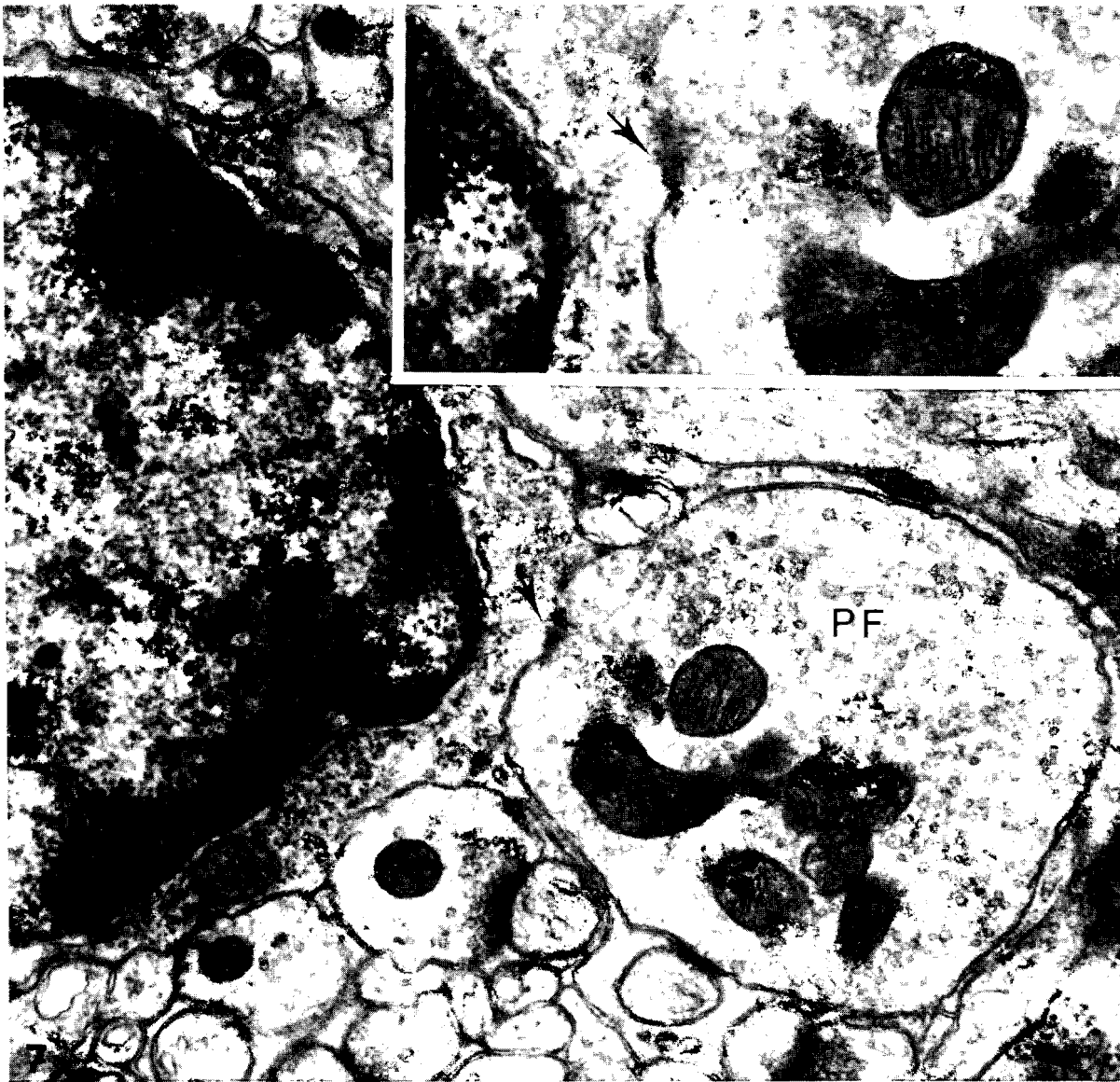
**Fig. 3.** Graphic representation of the number of EGCs per unit surface of the molecular layer of 30- and 180-day hypothyroid rats. \*  $p < 0.05$



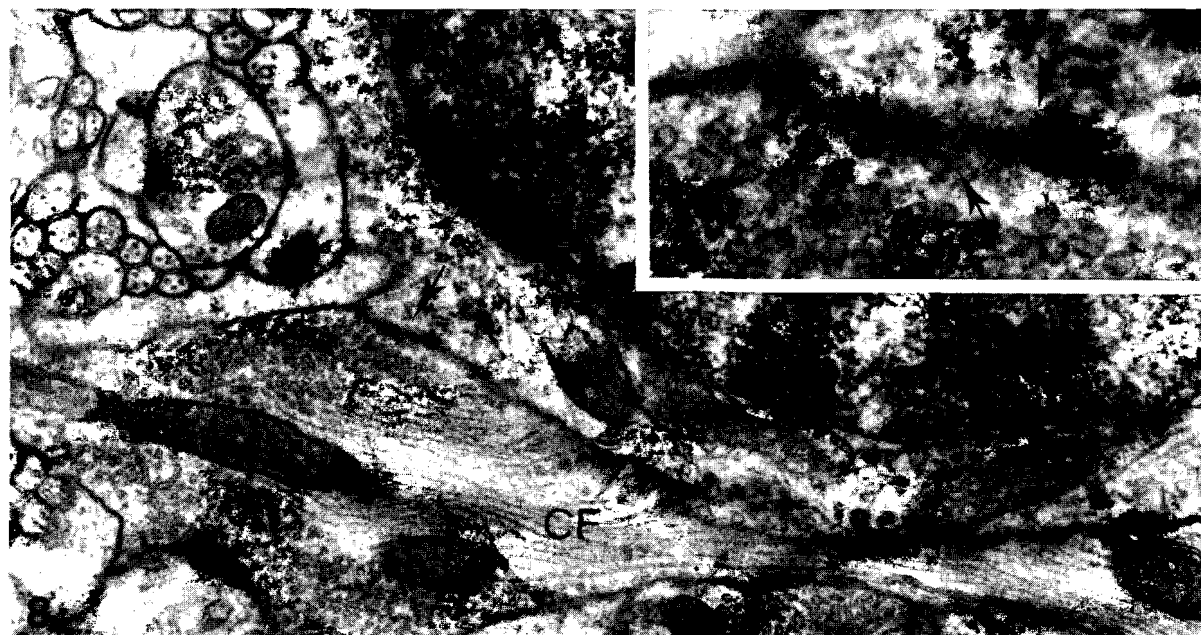
**Fig. 4.** EGCs from a 30-day hypothyroid rat. Dendrites are poorly developed (arrows)  $\times 400$ . Inset. Higher magnification of one of the granules. The characteristic digitiform dendritic terminals can be observed (arrow-heads).  $\times 810$

**Fig. 5.** EGCs from a 180-day hypothyroid rat, with vertically oriented dendrites (arrows).  $\times 400$ . Inset. Higher magnification of the deeper EGC with unbranched dendrites, displaying a club-shaped configuration and short terminal indentations (arrow-heads). Its ascending axon is seen arising from a dendrite (arrows).  $\times 800$

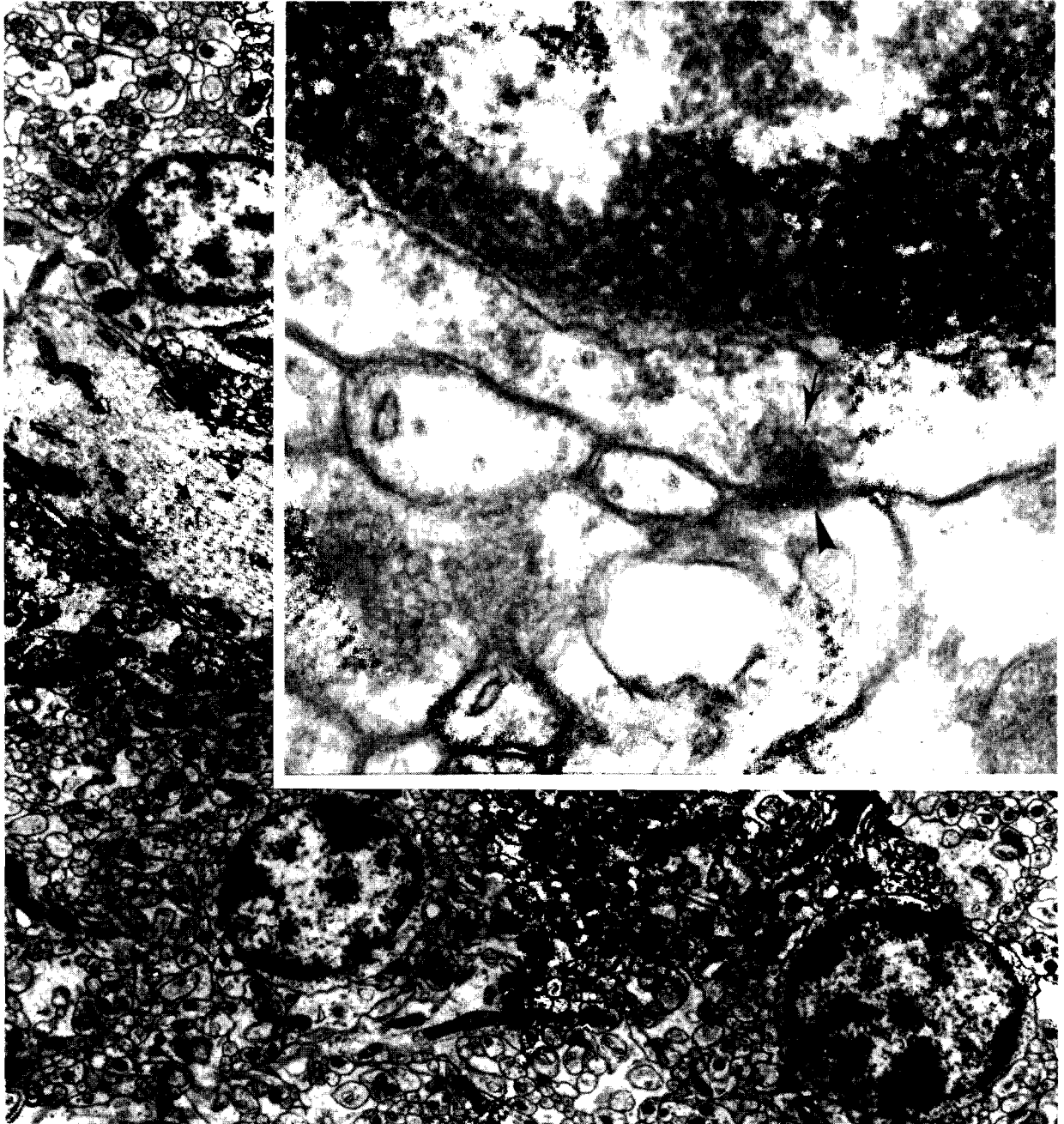
**Fig. 6.** EGC from a 180-day hypothyroid rat with vertically oriented dendrites (arrows).  $\times 400$ . Inset. Higher magnification of the EGC. A sinuous ascending axon (arrow-heads) with varicosities arising from a dendrite is seen.  $\times 800$



**Fig. 7.** Synaptic contact (arrow) between a parallel fiber bouton (PF) and an EGC in a 180-day hypothyroid rat.  $\times 30,000$ . Inset. Higher magnification of the synaptic contact. Round vesicles and a continuous post-synaptic density are seen.  $\times 50,000$



**Fig. 8.** Synaptic contact (arrow) between a climbing fiber (CF) and an EGC in a 180-day hypothyroid rat.  $\times 24,000$ . Inset. Higher magnification of the synaptic contact. Round vesicles and thick pre-synaptic densities (arrows) and post-synaptic densities (arrow-heads) are seen.  $\times 80,000$



**Fig. 9.** Three EGCs in the inner third of the molecular layer. A somatodendritic synapse between one granule cell and a Purkinje cell spine can be seen (arrow).  $\times 7,500$ . Inset. Higher magnification of the synaptic contact. Round vesicles (arrow) and thick, continuous post-synaptic densities (arrow-head) are recognized.  $\times 100,000$

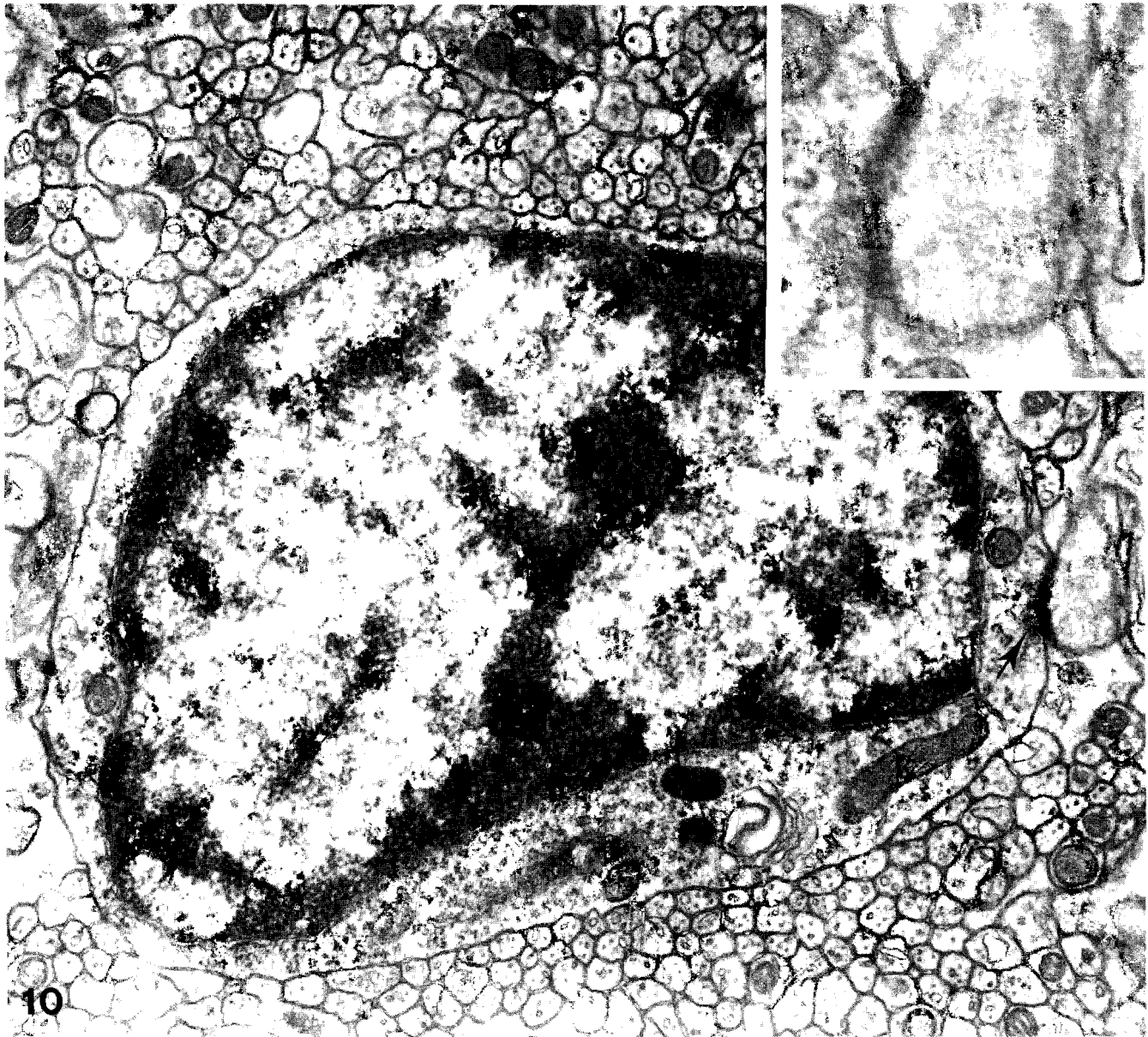


Fig. 10. EGC in the middle third of the molecular layer. A somatodendritic synapse with a Purkinje cell spine can be seen (arrow).  $\times 18,000$ . Inset. Higher magnification of the synaptic contact, which displays the same morphological features described in Fig. 9.  $\times 80,000$

exist between their dendritic trees and those of hypothyroid rats.

The number of dendrites displayed by EGCs (ranging from 1 to 3) was reduced (Figs. 4,5,6) when compared with those seen in normally positioned granule cells. Most frequently, dendrites were straight or slightly sinuous, unbranched and vertically oriented (Figs. 4,5,6). The characteristic claw shaped terminal ramification was poorly developed and the digitiform indentations were rarely seen (Fig. 4). In two cases, a

thin ascending axon was seen (Fig. 5).

#### *Ultrastructural observations*

Twenty EGCs from each control group and 50 EGCs from each hypothyroid group were studied.

The regular rim formed by the cytoplasm of EGCs was thinner than that seen in normally placed granule cells.

Synapses were never found between the perikaria of

granule cells and the molecular layer components of controls and 30-day-old hypothyroid rats.

On the contrary, numerous axosomatic synapses between parallel fibre boutons (Fig. 7) and climbing fibre enlargements (Fig. 8) with EGCs were seen; synaptic boutons displayed round vesicles and the post-synaptic densities were thick (Figs. 7,8). Besides, we observed 5 somatodendritic synapses (Figs. 9,10) between granule cell somas and Purkinje cell dendritic spines. This rare synaptic contact, first described in the hare cerebellar molecular layer (Spacek et al., 1973), showed round vesicles, prominent dense projections and continuous post-synaptic densities (Figs. 9,10).

The foci of EGCs located beneath the pial surface did not reveal any type of glomerular arrangement as described by other authors (Chan-Palay, 1972). Mossy fibre terminals invading the molecular layer were not disclosed either.

## Discussion

Among the numerous and outstanding observations made by Legrand and co-workers in the cerebellum of hypothyroid rats (see Legrand, 1982, for review), an increased cellularity in the molecular layer at post-natal day 28 was referred (Legrand, 1967). This increase was due to vertically-oriented and bipolar-shaped cell profiles, identified as migrating granule cells that remained visible until post-natal day 35. Their continued presence well-beyond the expected normal end of cerebellar granule cell histogenesis was interpreted as a consequence of an abnormal persistence of EGL (Legrand, 1967, 1982) and served as the basis for the attractive hypothesis that a prolonged neurogenesis in hypothyroid rats could act as a compensatory mechanism for the transient depression in the rate of the granular layer process of cell acquisition that would thus ultimately attain normal values (Balázs et al., 1971; Nicholson and Altman, 1972a; Legrand, 1982).

By using unbiased numerical estimations, we were, however, able to demonstrate that although the granule cell density did not significantly differ between controls and hypothyroid rats (Madeira et al., 1986, 1988), as previously pointed out by other researcher (Nicholson and Altman, 1972a; Clos and Legrand, 1973), there was a decrease in the total number of cells in the latter group due to a reduction of their granular layer volume (Madeira et al., 1986, 1988). We therefore suggested that the afore-mentioned prolonged granule cell neurogenic period in hypothyroid rats was not sufficient for a thorough compensation of the estimated 25% reduction of granule cells observed at the end of the second post-natal week (Legrand, 1967).

The results reported herein reinforce this assumption as they show that by day 30, EGCs appear as mature neurons and thus are unable to migrate. In fact, in semithin sections, most of their perikarya were seen as circular profiles scattered in the molecular layer and, moreover, their dendrites were identical to those seen in normally located cells, although they displayed an

impoverished branching pattern. On the basis of these morphological features we felt it sound to advance, as others have done in different circumstances (Landis, 1973), that a progressive structural complexity of the molecular layer could lead to an arrest of these cells during their migration towards the internal granular layer. This fits in nicely with the data obtained in 180-day-old hypothyroid rats where the dendritic morphology of EGCs did not differ from that of the 30-day-old group and where their number, although lesser than that of the latter group, remained considerably high. This would indicate that it is most unlikely that an effective ongoing migration process would take place by day 30, as advanced by other authors (Legrand, 1982).

On the other hand, the EGC synapses observed in the 180-day-old group, with parallel and climbing fibres, as well as the rare somatodendritic contacts between these cells and the spines of Purkinje cell dendrites, indicate that EGCs manage to adapt themselves to an adverse milieu (Landis, 1973; Mariani, 1977; Sotelo, 1978; Sotelo and Río, 1980). The presence of these synapses between current partners, but formed at an abnormal location (ectopic synapses) (Sotelo and Río, 1980), supports the interesting hypothesis advanced by Sotelo that neuronal differentiation depends not only on genetic factors but also on the cell environment (Sotelo and Río, 1980).

The capacity of EGCs to form ectopic synapses may also be related to the moderate numerical reduction in their number, observed when comparing the 30- and 180-day-old hypothyroid groups. In fact, it is well-known that neuronal afferents play an important role in cell differentiation and a continuous trophic activity upon their post-synaptic targets (Landis, 1973; Sotelo, 1975, 1978; Sotelo and Río, 1980) which might, in the present case, contribute to impede EGCs degeneration. On the other hand, it is worth noting that, rather unexpectedly, the tridimensional moulding of EGC dendritic trees and its synaptic investment were poorly affected by the lack of thyroid hormones despite the well-known fact that hypothyroidism markedly interferes with neurite outgrowth (Ruiz-Marcos et al., 1979; Legrand, 1982) and synaptogenesis (Nicholson and Altman, 1982b).

The presence of EGCs with somatodendritic synapses between their perikarya and Purkinje cell dendritic spines is thought to be related to a generalized impoverishment of cerebellar granule cells, according to observations made in tissue cultures (Kim, 1974) and in some mutant mice such as the weaver (Sotelo, 1975) and the reeler (Mariani et al., 1977). This assumption is in keeping with our previous results (Madeira, 1986, 1988) which showed that the number of granule cells in the cerebellum of hypothyroid rats is severely reduced.

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